Stomatal response of *Pinus sylvestriformis* to elevated CO_2 concentrations during the four years of exposure

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Abstract: Four-year-old *Pinus sylvestriformis* were exposed for four growing seasons in open top chambers to ambient CO_2 concentration (approx. 350 μmol·mol^{-1}) and high CO_2 concentrations (500 and 700 μmol·mol^{-1}) at Research Station of Changbai Mountain Forest Ecosystems, Chinese Academy of Sciences at Antu Town, Jilin Province, China (42°N, 128°E). Stomatal response to elevated CO_2 concentrations was examined by stomatal conductance (g_s), ratio of intercellular to ambient CO_2 concentration (c_i/c_a) and stomatal number. Reciprocal transfer experiments of stomatal conductance showed that stomatal conductance in high-[CO_2]-grown plants increased in comparison with ambient-[CO_2]-grown plants when measured at their respective growth CO_2 concentration and at the same measurement CO_2 concentration (except a reduction in 700 μmol·mol^{-1} CO_2 grown plants compared with plants on unchambered field when measured at growth CO_2 concentration and 350 μmol·mol$^{-1}CO_2$). High-[CO_2]-grown plants exhibited lower c_i/c_a ratios than ambient-[CO_2]-grown plants when measured at their respective growth CO_2 concentration. However, c_i/c_a ratios increased for plants grown in high CO_2 concentrations compared with control plants when measured at the same CO_2 concentration. There was no significant difference in stomatal number per unit long needle between elevated and ambient CO_2. However, elevated CO_2 concentrations reduced the total stomatal number of whole needle by the decline of stomatal line and changed the allocation pattern of stomata between upper and lower surface of needle.
Key words: c_i/c_a ratio; High CO_2; *Pinus sylvestriformis*; Stomatal conductance; Stomatal number; Stomatal line
Abbreviations: g_s, *stomatal conductance;* c_i, *intercellular CO_2 concentration;* c_a, *ambient CO_2 concentration*
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Introduction

Stomata directly affect the gas exchange of CO_2/H_2O between atmosphere and foliage. The impact of elevated CO_2 concentration on the stomatal behavior has attracted considerable attention. The stomatal response to CO_2 is important in understanding stomatal physiology, and vegetation-atmosphere exchanges at all scales from the individual plant up to global vegetation (Morison 1998). Short-term and long-term effects of increased CO_2 on stomata are different. In general, the short-term response of stomata is a change in aperture (usually reversible), and long-term response includes anatomical and morphological changes, for example, in stomatal number and/or in size (Morison 1998). Stomatal acclimation may occur when plants are exposed to increased CO_2 concentration for a long time.

Stomatal conductance (g_s) is most frequently used for assessing the function of stomata in reconciling the water loss and carbon gain (Zhang *et al.* 2002). It is generally accepted that an increase in the ambient CO_2 concentration can cause reductions in stomatal conductance resulted from the decrease of stomatal aperture and/or density, and the reduction varied widely (Bunce 2000; Morison 2001). A reduction in stomatal conductance is a common response of herbaceous plants to elevated CO_2 (Bunce 2000). Some experimental evidences suggested that many forest tree species show small or non-significant change in stomatal conductance under long-term elevated CO_2 (Curtis 1996; Saxe *et al.* 1998), particular conifer (Teskey 1995).

Stomata appear to response directly to the intercellular CO_2 concentration (c_i), rather than ambient CO_2 concentration (c_a), as demonstrated by Mott (Mott 1988). C_3 plants normally maintain relative constancy of the ratio of intercellular to ambient CO_2, approx. 0.7 (Lodge *et al.* 2001). Given no adjustment of stomata, the rate of CO_2 diffusion through the stomatal pores would rise in proportion to the increase in ambient CO_2 (Jarvis *et al.* 1999). Therefore, whether c_i/c_a ratio remains constant with increased c_a should be examined carefully.

The change of stomatal number of needle is a long-term response to elevated CO_2 concentration. Some literatures have reported no change in stomatal density (Poole *et al.* 2000; Lodge *et al.* 2001). There was no difference in stomata density for current-year needles of Sitka spruce trees exposed to elevated CO_2 concentration for 4 years between treatments (Barton and Jarvis, 1999), the same phenomenon also was observed on *Alnus glutinosa* (Poole *et al.* 2000).

The main objectives of this study are to determine stomatal response of *Pinus sylvestriformis* to long-term exposure to high CO_2 concentration: (a) to determine g_s and c_i/c_a ratio at different measurement of CO_2 concentration; (b) to examine the changes of stomatal number of current-year needle.

Materials and methods

The study site is located at Research Station of Changbai Mountain Forest Ecosystems, Chinese Academy of Sciences at Antu Town, Jilin Province, China (42°N, 128°E). Average annual rainfall is 700 mm. In 1999, seedlings of *Pinus sylvestriformis* were planted in open top chambers and on un-chambered field. Open top chamber consists of aluminium frames of 1.2 m in length, 0.9 m in width and height, and clear glass covers. CO_2 enters the chamber through perforated plastic pipe at the bottom

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of chamber. Fan is hung in the top of the chamber to mix the gas well-proportioned. Treatments consist of three concentration levels of CO_2: ambient, 500 and 700 μmol·mol^{-1} CO_2. Seedlings in the control chamber and on un-chambered field are given ambient CO_2, approx. 350 μmol·mol^{-1}. The CO_2 concentrations in each chamber were checked weekly and adjusted. Elevated CO_2 concentrations were provided by the mixture of industrial high CO_2 and ambient CO_2. 500 and 700 μmol·mol^{-1} CO_2 were obtained by adjusting the velocity and amount of flow of industrial CO_2 and ambient CO_2. CO_2 concentration was monitored by the CI—301 gas analyzer once a week. The plants were four years old, with average height of 47 cm, which are daily irrigated except rainy day.

All measurements were made on the current-year needle. The measured needles were near the top crown and received the full sunlight. The experiment was begun after the plants had been exposed to the CO_2 treatments for 3 months in the fourth growing season. Seedlings had been treated by high CO_2 concentrations (500 and 700 μmol·mol^{-1} CO_2) continuously (24 h·d^{-1}) during growing season from June to September since 1999.

Measurement of stomatal conductance and c_i/c_a ratio

Stomatal conductance (g_s) and c_i/c_a ratio were measured with a portable photosynthetic analyzer equipped with a conifer cuvette (LI6400, Li-Cor, Inc., Lincoln, NE). Reciprocal transfer experiment of g_s and c_i/c_a in high- and low-[CO_2]-grown plants was carried out at three levels of CO_2 concentrations (350, 500 and 700 μmol·mol^{-1}) in the cuvette, respectively. All measurements were made directly under light saturating conditions. The readings were taken after allowing g_s to reach a steady state.

Stomatal number

Twenty needles were collected at random from 20 plants per treatment. Stomatal number was separately counted on the upper and lower surface of current needles. Upper and lower surface of each needle were cut down 3-mm long epidermis along the needle, which was viewed with a microscope. The number of all stomatal lines and number of stomata per line on 3-mm long epidermis were counted.

Statistics

Mean values of stomatal conductance and c_i/c_a ratio were compared separately. One-way analysis of variance was performed for three comparisons. One contrast was carried out to compare needles grown and measured at 350 μmol·mol^{-1} CO_2 with those grown at 700 and 500μmol·mol^{-1} CO_2 but measured at 350 μmol·mol^{-1} CO_2. The second contrast was to compare needles grown and measured at 500 μmol·mol^{-1} CO_2 with those grown at 700 and 350 μmol·mol^{-1} CO_2 but measured at 500 μmol·mol^{-1} CO_2. The third contrast was to compare needles grown and measured at 700 μmol·mol^{-1} CO_2 with those grown at 500 and 350 μmol·mol^{-1} CO_2 but measured at 700 μmol·mol^{-1} CO_2. Stomatal line and stomatal number on upper, lower surface and whole needle were compared among the four treatments. All statistical tests were performed using SPSS 11.5 software. The conclusions were reached by the LSD tests.

Results

Reciprocal transfer experiment of g_s

Stomatal conductance (g_s) of *Pinus sylvestriformis* in the 500 μmol·mol^{-1} CO_2 was 61% and 4% higher than those in control chamber and on un-chambered field when measured at their respective growth CO_2 concentrations. Similarly, g_s in 700 μmol·mol^{-1} CO_2 increased by 11% and decreased by 28%, compared with those of growing in the control chamber and on un-chambered field, respectively (Table 1). The difference was significant between elevated CO_2 and ambient CO_2 ($p<0.05$).

When measured at 500 or 700 μmol·mol^{-1} CO_2, stomatal conductance of *Pinus sylvestriformis* at elevated CO_2 concentrations was substantially higher than those at ambient CO_2 concentration. g_s at 700 μmol·mol^{-1} CO_2 was 22% lower than that on un-chambered field when measured at 350 μmol·mol^{-1} CO_2. g_s at 500 μmol·mol^{-1} CO_2 was the highest at any measurement CO_2 concentration. g_s of both high-[CO_2]-grown and control plants declined with the increase of measurement CO_2 concentration.

Table 1. Mean stomatal conductance (mol·m^{-2}·s^{-1}) in *Pinus sylvestriformis* grown at ambient CO_2 and elevated CO_2 concentrations measured at three different CO_2 concentrations (350, 500, and 700 μmol·mol^{-1} CO_2)

Growth conditions (μmol·mol^{-1} CO_2)	Measurement CO_2 concentration (μmol·mol^{-1})		
	350	500	700
700	0.284±0.001	0.273±0.0002	0.262±0.0004
500	0.399±0.001	0.379±0.001	0.371±0.002
Control chamber (350)	0.235±0.001	0.216±0.0003	0.206±0.0003
Un-chambered field (350)	0.365±0.004	0.235±0.001	0.181±0.003

Note: Values shown above are means ± standard error. Comparisons were made among the four treatments at each measurement CO_2 concentration. Results for a one-way analysis of variance showed the difference were significant ($p<0.05$).

Reciprocal transfer experiment of c_i/c_a ratio

When measured at their respective growth CO_2 concentration, high-[CO_2]-grown plants exhibited lower c_i/c_a ratios compared eith the control plants (Table2). c_i/c_a ratio of *Pinus sylvestriformis* grown at 700 μmol·mol^{-1} CO_2 was 5% and 3% lower than those at control chamber and un-chambered field, respectively. It was 4% and 2% lower than the control chamber and un-chambered field for *Pinus sylvestriformis* grown at 500 μmol·mol^{-1} CO_2. The difference was significant between elevated CO_2 and ambient CO_2. However, the c_i/c_a ratio increased for *Pinus sylvestriformis* grown in high CO_2 concentrations when exposing to 350 μmol·mol^{-1} CO_2.

Table 2. c_i/c_a ratio of *Pinus sylvestriformis* grown at ambient and elevated CO_2 concentrations measured at three different CO_2 concentrations (350, 500 and 700μmol·mol^{-1} CO_2), respectively

Growth conditions (μmol·mol^{-1} CO_2)	Measurement CO_2 concentration (μmol·mol^{-1})		
	350	500	700
700	0.719±0.003	0.685±0.001	0.667±0.001
500	0.728±0.001	0.676±0.001	0.693±0.001
Control chamber (350)	0.701±0.002	0.635±0.002	0.600±0.001
Un-chambered field (350)	0.687±0.002	0.686±0.0003	0.660±0.002

Note: Values shown above are means ± standard error. Comparisons were made among the four treatments at each measurement CO_2 concentration. Results for a one-way analysis of variance showed the difference were significant except the comparison between 700 μmol·mol^{-1} CO_2 and un-chambered field at 500 μmol·mol^{-1} CO_2 measurement CO_2 concentration ($p<0.05$).

When control plants grown at ambient CO_2 concentration were measured at high CO_2 concentrations (500 and 700 μmol·mol^{-1} CO_2) the c_i/c_a value decreased. The c_i/c_a ratios of high-[CO_2]-grown plants were higher than those of control plants when measured at the same CO_2 concentration.

Stomatal number

Stomata of *Pinus sylvestriformis* occur in a few of straight lines running along the length of the needle on both sides of the needle. The number of stomatal line per needle (including upper and lower surface) at high CO_2 concentrations was significantly lower than that on un-chambered field (Table 3). The number of stomatal line at 700 μmol·mol^{-1} CO_2 approximately equals to that at 500 μmol·mol^{-1} CO_2. The number of stomatal line of plants grown on un-chambered field was higher than that in the control chamber though both accepted ambient CO_2 concentration. The stomatal line of plants in the control chamber was 10% higher than those at 700 and 500 μmol·mol^{-1} CO_2. But there was no significant differences between control chamber and elevated CO_2 concentrations. The allocation of stomatal line and number of stomata was different between upper and lower surface of needle for high- and low-[CO_2]-grown plants. Stomatal line and number of stomata on upper surface were more than those on lower surface. The number of stomatal line on the upper surface of needle grown at 700 and 500 μmol·mol^{-1} CO_2 decreased by 16% and 8%, respectively, compared with that in the control chamber,. Similarly, the number of stomatal line on the upper surface of needle grown at 700 and 500 μmol·mol^{-1} CO_2 decreased by 25% and 19%, respectively, compared with that on un-chambered field. The number of stomatal line on the lower surface of needle grown at 700 μmol·mol^{-1} CO_2 showed no reduction and was 11% lower grown at 500 μmol·mol^{-1} CO_2, compared with that in the control chamber. *Pinus sylvestriformis* grown at 700 and 500 μmol·mol^{-1} CO_2 exhibited that the numbers of stomatal line on the lower surface of needle were 11% and 23% separately lower than that at the un-chambered field. There was no significant difference on the number of stomata on lower surface among four treatments. Stomatal number per unit long needle on upper surface at 700 μmol·mol^{-1} CO_2 was much higher (increased by 16%) than that on the un-chambered field. However, elevated CO_2 did not significantly change the total stomatal number (including upper and lower surface). The results of variance on the stomatal line and stomatal number among four treatments were shown at Table 4.

Table 3. Stomatal line and stomatal number of current-year needle of *Pinus sylvestriformis* exposure to high CO_2 concentrations for four growing seasons

Indexes	Growth CO_2 concentration (μmol·mol^{-1} CO_2)			
	700	500	Control chamber (350)	Unchambered field (350)
Number of stomatal line on upper surface of needle	7.6±0.413	8.3±0.442	9.0±0.397	10.2±0.485
Number of stomata 1mm long needle on upper surface	12.1±0.252	11.4±0.249	11.7±0.246	11.3±0.163
Number of stomatal line on lower surface of needle	6.3±0.448	5.5±0.256	6.2±0.296	7.1±0.352
Number of stomata 1mm long needle on lower surface	10. 8±0.171	10.7±0.229	11.0±0.256	10.8±0.183
Number of stomatal line per needle	13.9±0.737	13.8±0.627	15.2±0.601	17.3±0.781
Number of stomata 1mm long needle	22.9±0.359	22.1±0.389	22.6±0.443	22.2±0.294

Table 4. Results of one-way analysis of variance of stomatal line and stomatal number (P: 0.05 level)

	1-2	1-3	1-4	2-3	2-4	3-4
Number of stomatal line on upper surface of needle	0.227	0.021	0	0.259	0.003	0.055
Number of stomata 1mm long needle on upper surface	0.056	0.231	0.034	0.464	0.832	0.345
Number of stomatal line on lower surface of needle	0.106	0.838	0.129	0.156	0.002	0.086
Number of stomata 1mm long needle on lower surface	0.693	0.537	0.923	0.315	0.766	0.476
Number of stomatal line per needle	0.959	0.171	0.001	0.156	0.001	0.039
Number of stomata 1mm long needle	0.156	0.591	0.167	0.385	0.971	0.405

Note: 1: 700 μmol·mol^{-1} CO_2; 2: 500 μmol·mol^{-1} CO_2; 3: control chamber; 4: un-chambered field

Discussion

Stomatal conductance, ratio of intercellular to ambient CO_2 concentration and stomatal number are main parameters of assessing stomatal behavior at elevated CO_2 concentration. Stomatal conductance can well describe the dynamic changing trend of stomatal characteristics, while relative stable properties can be provided by stomatal number (Zhang *et al.* 2002).

It is widely stated that elevated CO_2 concentration will cause the reduction of stomatal conductance. Stomatal conductance is affected primarily by stomatal aperture and the number of stomata i.e. stomatal density (Weyers and Lawson 1997). Thus changes in size and number of stomatal aperture play key roles in stomatal conductance. Since stomatal number of *Pinus sylvestriformis* was decreased by elevated CO_2 concentrations, the increase of stomatal conductance at elevated CO_2 concentrations mainly related to stomatal aperture. *Pinus sylvestriformis* grown at 500 μmol·mol^{-1} CO_2 showed the highest stomatal conductance at any measuring CO_2 concentration. However, the change of stomatal conductance of *Pinus sylvestriformis* grown at 700 μmol·mol^{-1} CO_2 was related to the measuring CO_2 concentration. Stomata are sensitive to some environmental stimuli, particular light, humidity and CO_2. Therefore, changes of stomatal conductance in 700 μmol·mol^{-1} CO_2 could be caused mainly by the change of environmental CO_2 concentration. Therefore, the simplest interpretation is that the difference in stomatal conductance between high- and ambient-[CO_2]-grown plants was a result of the direct adjustment of stomatal aperture.

The change of stomatal aperture or conductance could affect

the c_i/c_a ratio of stomata which are directly sensitive to intercellular CO_2 concentration (Mott, 1990). g_s and c_i/c_a ratio in high-[CO_2]-grown plants were higher than those of control plants when measured at high CO_2 concentrations, confirming that stomata of *Pinus sylvestriformis* acclimated to long-term exposure to high CO_2 concentrations. High-[CO_2]-grown plants showed a decrease for the ratio of c_i/c_a compared with control plants when measured at their respective CO_2 concentration of growth. The decrease of c_i/c_a ratio in high-CO_2-grown plants was mainly caused by higher ambient CO_2 concentration (700 and 500 $\mu mol \cdot mol^{-1}$ CO_2).

Given no adjustment to the change of increasing atmospheric CO_2 concentration the c_i/c_a ratio would remain constant. However, Ellsworth (1999) found a similar tendency in c_i/c_a ratio for well-watered *P. taeda*. Sage (1994) and Drake *et al.* (1997) also demonstrated it in many experiments, including those in which plants were grown for long periods in high CO_2 concentration. But it is surprising that the ratio of c_i/c_a unchanged at high CO_2 concentration. Sage (1994) found that except under water and humidity stress, c_i/c_a exhibited inconsistent change in a variety of C_3 species. In our study, high-[CO_2]-grown *Pinus sylvestriformis* exhibited that c_i/c_a ratio was below the control plants when measured at their respective growth condition, as also demonstrated by Wong (1993). c_i/c_a ratio increased in high-[CO_2]-grown plants when measured under 700 and 500 $\mu mol \cdot mol^{-1}$ CO_2, confirming that stomata do not always maintain c_i/c_a constant.

Stomatal number of current-year needle of *Pinus sylvestriformis* decreased under 700 and 500 $\mu mol \cdot mol^{-1}$ CO_2, and the allocation pattern of stomata between upper and lower surface of needle gave rise to change. Stomatal lines on the upper surface of needle showed larger reduction at 700 $\mu mol \cdot mol^{-1}$ CO_2, and that on the lower surface showed larger decline at 500 $\mu mol \cdot mol^{-1}$ CO_2. The difference was not significant though the total stomatal lines of whole needle (including upper and lower surface) in the control chamber were higher than that in elevated CO_2. The total stomatal lines of needle on un-chambered field were remarkably higher than those in the open top chambers (including 700, 500 $\mu mol \cdot mol^{-1}$ CO_2 and control chamber). Therefore, the microenvironment of open top chamber can affect the number of stomatal line of *P. sylvestriformis*. That is both elevated CO_2 and microenvironment of open top chamber decreased the number of stomatal line, but they did not change the stomatal number per unit long needle. Therefore, the total stomatal number of *P. sylvestriformis* had a decrease at 700 and 500 $\mu mol \cdot mol^{-1}$ CO_2 by decreasing stomatal line on upper and lower surface of needle. The change of stomatal number is a result of long-term exposure to high CO_2 concentrations. The decline of stomatal number for *Pinus sylvestriformis* did not significantly affect the change of stomatal conductance. Some studies showed stomata density did not change at high CO_2 concentration.

There was difference in stomatal response for *Pinus sylvestriformis* to 700 and 500 $\mu mol \cdot mol^{-1}$ CO_2. The increases of stomatal conductance and c_i/c_a ratio of plants grown at 500 $\mu mol \cdot mol^{-1}$ CO_2 were relatively bigger than those at 700 $\mu mol \cdot mol^{-1}$ CO_2. In addition, the allocation of stomatal line on upper and lower surface was different, too. Stomatal behavior of plants in the control chamber was also different from that on un-chambered field. By this study, we found that the microenvironment of open-top chamber affected the physiological characteristics of *Pinus sylvestriformis*. But we still do not confirm which factors and how these factors operate.

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